DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Michael Watson, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	30-Day Dietary Toxicity in the Rodent
MRID NO:	447343-03
CHEMICAL NO:	006466
TEST MATERIAL:	Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*
STUDY NO:	RIKILT-DLO No. 71.113.04.D97.4
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	RIKILT-DLO, Netherlands
TITLE OF REPORT:	Mouse Short-Term (30-Day) Dietary Toxicity Study with the Protein Cry9C
AUTHOR:	Sally Van Wert, Ph.D.; Dr. H.P.J.M. Noteborn
STUDY COMPLETED:	4 September 1998
CONCLUSION:	All animals appeared healthy, survived to termination of the study, and generally gained weight. No significant differences were seen in the parameters measured for clinical chemistry or hematology. The pathologist reported increased leanness in high dose treated mice seen in both the superficial tissues and abdominal viscera (10/12 animals). The hearts in the treated groups were also noted as having surface hemorrhages in 5/12 and 9/12 animals in the low and high dose Cry9C treatments, respectively. No unusual findings were made of any of the histopathology sections examined. The immunocytological examination of the GI tract found no binding of the the Cry9C protein to villi or enterocytes lining the crypts of both the large and small intestines. Lymphatic tissue of the intestines (i.e., Peyer's patches), the spleen, submandular glands, mesenteric lymph nodes and thymus were all normal upon microscopic examination. Western blot analysis of fecal contents did find Cry9C in a degraded form (55kDa). There was an apparent change in the fat content of the high Cry9C dose group which was seen in a subjective determination of decreased fat pad size. The 28 day (and only) urinalysis of the high dose Cry9C group also indicated an elevated ketone level. Both these findings indicate some perturbation in the fat metabolism of the high dose group which has unclear toxicological significance.
CLASSIFICATION:	Acceptable. The dietary exposure study was not required for this active ingredient.

STUDY DESIGN

This study was not done according to the GLP standards found in 40CFR160 or the OECD Council Decision C(81)30 of 12 May 1981. The report is also being claimed as confidential by RIKILT-DLO but not by AgrEvo. AgrEvo's statement on data confidentiality also states that AgrEvo's claim

supercedes all other confidentiality statements in the report. The study was an oral 28-day exposure to Cry9C protein through drinking water.

Test material: The test substance was a purified trypsinized form of the Cry9C protein (Lys164Arg) as expressed in a *Bacillus* strain with the cry9C.PGS1a gene. The test substance was provided by Plant Genetic Systems in two lyophilized batches which were run on SDS-PAGE to confirm their composition/purity. The protein content was determined by a BCA Protein Assay (Pierce Chemicals, Rockford, Il) with BSA as the standard.

Test animals: Forty-three ♀ mice (NMRI outbred) were obtained from a specific pathogen free colony at Harlan Germany and weighed between 27.6 and 32.8g at receipt. After isolation and acclimatization, thirty-six mice were chosen for use in the study based on their weight gains from receipt and bodyweight variation being less than 15% of the 29.0g mean. The animals were identified by ear tag and kept two per cage during the study. For two days over the 28-day test period, the animals were kept in metabolic cages. Otherwise they were maintained in the Central Experimental Animal Accommodation under standard conditions (12 hours artificial light/dark at 21±1 °C with a controlled humidity of 55±10 % which only rose during the weekly cleaning to 80-90 % RH for approximately one hour). The mice received the Institute's basal rodent diet (RMH-B) and liquid *ad libitum*. Tap water (supplemented with test substance for the treated groups) was provided in glass bottles which were refilled daily with fresh solution.

Test methods:
Dosing: The test substance was provided to the mice in their water. The test substance doses amounts are based on a daily consumption of 5.0 ml water/30.0 gm mouse. The provided solutions were at 0.21 or 2.1 gm Cry9C/l for a calculated dosing amount of 35 or 350 mg/kg. The dosing was begun October 6 and 7, 1997 and terminated November 4 and 5, 1997. Fifteen samples from the drinking water bottles were taken throughout the dosing regime after standing at room temperature for 24 hours. These samples were subjected to a western blot analysis to confirm the composition and stability of Cry9C in the dosing solution. The weekly group mean of the Cry9C intake was monitored by tracking body weight and liquid consumption. The individual animals were weighed weekly, water consumption per cage of two animals was recorded daily and the food consumption monitored by weight weekly. Feed utilization efficiency (%) was calculated by tracking the group mean body weight gain (g/animal/day) divided by the group mean food consumption (g/animal/day) x 100.

Clinical Signs and Biochemical Monitoring: The general condition and behavior of the animals was examined twice daily on weekdays and once daily on the weekends. On day 5 before the start of the dosing and on day 29 blood samples were collected from the orbital sinus and subjected to routine hematology including the following: hemoglobin, red and white blood cell count, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, and reticulocyte, neutrophil, lymphocyte, monocyte, eosinophil and basophil cell counts. On day 30 additional blood chemistry was performed on samples taken examining the following: alkaline phosphatase, total protein, albumin, bilirubin, urea nitrogen, creatine, glucose, cholesterol, calcium, serum glutamic oxaloacetic transaminase and hemoglobin. On day 28 the mice were placed in metabolic cages to

collect urine for routine urinalysis which included: volume, pH, protein, glucose, blood, ketone, and bilirubin content.

An ELISA was run on the blood serum and 24 hour feces samples to determine Cry9C levels present. The 24 hour feces sample taken during the metabolic caging was frozen, diluted 1:1 in 50mM TrisHCL (pH7.3) with 1.0mM PMSF and 2.0mM EDTA. The extract was allowed to sit for one hour on ice before the supernatant was diluted 1:1 with 10mM Tris-HCl (pH8.0) with 1.0mM EDTA, 20% glycerol, 5% SDS, 2% dithiothreitol and 0.2% bromophenol blue. This solution was then boiled for 5 minutes and stored at -40°C until western blot analysis was performed.

After sacrifice on day 30, all animals received a gross pathological examination with a bone marrow smear of the femur being made at the same time. The weights of the following organs were recorded: adrenal, brain, heart, kidneys, liver, lungs, ovaries, spleen and thymus. Samples of the following organs were taken and preserved in formalin: brain, caecum, colon, femur with joint, heart, kidneys, liver, lungs, esophagus, ovaries, pancreas, rectum, thigh muscle, skin, small intestines, spleen, stomach, thymus, urinary bladder, uterus and any other tissue with gross macroscopic abnormalities. Sections of the following tissues were made, stained in eosin/Haematoxylon and examined by a pathologist: bladder, mesenteric lymph nodes, brain and spinal cord, caecum, colon, duodenum, heart and thyroid/trachea, ileum, jejunum with Peyer's patches, kidneys and adrenal glands, liver and lungs, esophagus and stomach, ovaries, rectum, spleen and thymus and uterus.

RESULTS AND DISCUSSION

Test material

The test material analyzed by PGS indicated that Batch I consisted of 100% Lys mutant Cry9C protein (MW 68kDa) and Batch II, approximately 70%. Both batches were approximately 1.1g in size. The remaining 30% of Batch II was said to contain untrypsinized protoxin, inactivated trypsin and inactivated Cry9C degradation products. RIKILT-DLO repeated a western blot and scanning densitometry analysis of Batch II to verify that 70-75% of the material was indeed Cry9C (68kDa). The testing lab also verified that the test substance was stable in solution for one day with spontaneous degradation to the 55kDa form.

Dose verification and food consumption

The uptake of the test substance was monitored by the daily water consumption which showed no differences among the treated groups and the controls. Therefore, the two treatment groups, based on average daily consumption of test substance amended water, received 33.3 $\pm$0.3mg or 328.0$\pm$4.0mg Cry9C per kg bodyweight per day. There was no effect on food consumption related to treatment group.

Bodyweights and Clinical Signs

All animals appeared healthy, survived to termination of the study, and generally gained weight. There were transient weight losses which were similar across treatment and control groups. No treatment related effects on body weight could be seen although individual animal data was not provided. No treatment groups gained more than 2.3g (~7% gain) from the initial weight during the course of the study. All animals lost bodyweight beginning day 28 and 29 when the groups were

being held in metabolic cages. Up to that date all the animals had been gaining weight at a similar rate.

Clinical Chemistry, Urinalysis and Hematology

No significant differences were seen in the parameters measured for clinical chemistry or hematology. Calcium and SGOT were not measured due to degradation or treatment of the blood samples. Decreased urinary volume and increased ketone were noted in urine samples from the high dose Cry9C treated animals taken in the metabolic cages. No Cry9C was found in the blood by ELISA.

Autopsy and Histopathology

Minimal common gross pathological findings were noted among the treatment groups such as pale kidneys, spotted spleen and lungs. The pathologist reported increased leanness in high dose treated mice seen in both the superficial tissues and abdominal viscera (10/12 animals). The hearts in the treated groups were also noted as having surface hemorrhages in 5/12 and 9/12 animals in the low and high dose Cry9C treatments, respectively. No unusual findings were made of any of the histopathology sections examined. The immunocytological examination of the GI tract found no binding of the Cry9C protein to villi or enterocytes lining the crypts of both the large and small intestines. Lymphatic tissue of the intestines (i.e., Peyer's patches), the spleen, submandular glands, mesenteric lymph nodes and thymus were all normal upon microscopic examination. Western blot analysis of fecal contents did find Cry9C in a degraded form (55kDa).

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